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Review Article

Pathogenetic role of eNOS uncoupling in cardiopulmonary disorders

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ABSTRACT

The homodimeric flavohemeprotein endothelial nitric oxide synthase (eNOS) oxidizes L-arginine to L-citrulline and nitric oxide (NO), which acutely vasodilates blood vessels and inhibits platelet aggregation. Chronically, eNOS has a major role in the regulation of blood pressure and prevention of atherosclerosis by decreasing leukocyte adhesion and smooth muscle proliferation. However, a disturbed vascular redox balance results in eNOS damage and uncoupling of oxygen activation from L-arginine conversion. Uncoupled eNOS monomerizes and generates reactive oxygen species (ROS) rather than NO. Indeed, eNOS uncoupling has been suggested as one of the main pathomechanisms in a broad range of cardiovascular and pulmonary disorders such as atherosclerosis, ventricular remodeling, and pulmonary hypertension. Therefore, modulating uncoupled eNOS, in particular eNOS-dependent ROS generation, is an attractive therapeutic approach to preventing and/or treating cardiopulmonary disorders, including protective effects during cardiothoracic surgery. This review provides a comprehensive overview of the pathogenetic role of uncoupled eNOS in both cardiovascular and pulmonary disorders. In addition, the related therapeutic possibilities such as supplementation with the eNOS substrate L-arginine, volatile NO, and direct NO donors as well as eNOS modulators such as the eNOS cofactor tetrahydrobiopterin and folic acid are discussed in detail.

Contents

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Introduction

It has been 30 years since Furchgott and Zawadski demonstrated that the endothelium produces a mysterious "endothelium-derived relaxing factor," which is responsible for vascular smooth muscle relaxation [1]. This was followed by the remarkable observation that this factor was the freely diffusible gas nitric oxide (NO) [2]. Today, NO is regarded as one of the body's most versatile molecules. It is a neurotransmitter, second messenger, inflammatory marker, and therapeutic agent that is generated by nitric oxide synthases (NOS). NOS catalyze the conversion of the amino acid L-arginine[3] and molecular oxygen to L-citrulline and NO, aided by cofactors. Three NOS isoforms have been identified, i.e., neuronal NOS (NOS-I), inducible NOS (NOS-II), and endothelial NOS (NOS-III; eNOS).

Endothelial NOS, as its name suggests, is mainly found in the endothelial lining of the blood vessels but also in the cardiomyocytes [4], airway epithelium [5], tubular cells of the kidney [6], and other organ systems. The cardiovascular system relies on eNOS for optimal function, most notably with NO as the principle mediator of flow-mediated dilation. In addition to blood pressure regulation, eNOS-derived NO is also responsible for inhibition of platelet aggregation, leukocyte adhesion, and smooth muscle cell proliferation (see Fig. 1 for the structure of eNOS) [7]. Consequently, functional impairment of this enzyme may result in endothelial dysfunction, leading to both pulmonary and systemic hypertension. Most actions of NO are mediated via the production of cGMP by guanylate cyclase, resulting in a decreased intracellular calcium (Ca^{2+}) concentration. Lower intracellular Ca^{2+} results in relaxation of the vascular smooth muscle layer and ultimately in vasodilatation and a decrease in blood pressure.

In this review, we discuss the molecular basis of eNOS uncoupling, the regulation of eNOS activity, its physiological functions in the cardiopulmonary system, and its role in the physiopathology of various cardiovascular and pulmonary diseases.

Molecular structure and function

eNOS is a homodimer that binds a number of different cofactors, which are required to convert L-arginine and O_2 to L-citrulline and NO. Each eNOS monomer has a bidomain structure. The N-terminus comprises the oxygenase domain and contains tetrahydrobiopterin (BH_4), heme iron, and L-arginine binding sites. L-Arginine and BH_4 promote enzyme dimerization and both act as a stabilizer of the active dimeric form [8,9]. The heme group is also essential for dimerization [10]. Further stabilization is generated by a zinc thiolate (ZnS_4) cluster formed by a zinc ion between two cysteine residues from each monomer. This cluster is responsible for the integrity of the BH_4 binding site [11]. The C-terminus is the reductase domain with binding sites for two flavins, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), and NADPH [12]. Electrons are produced by oxidation of NADPH to $NADP^+$ at the flavin domain of each monomer [12]. These electrons are then transferred, one at a

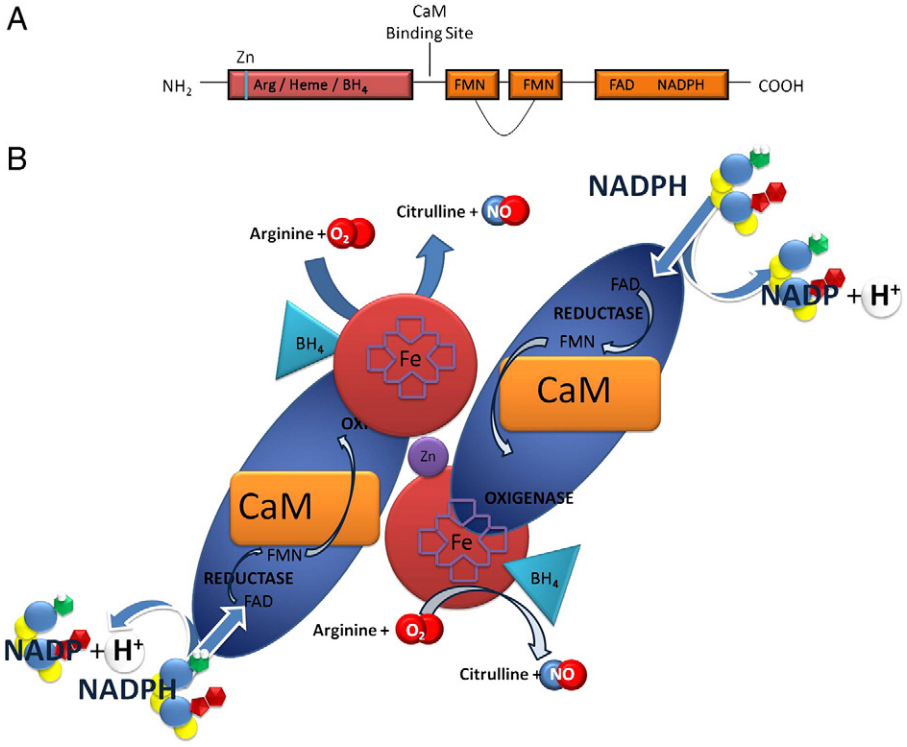

Fig. 1. Molecular structures of eNOS monomer and dimer. (A) Secondary structure of the eNOS monomer. The oxygenase (N-terminal) and reductase (C-terminal) domains are separated by a calmodulin (CaM) binding site. (B) Detail of the eNOS dimer. The Zn ion is responsible for connecting the monomers at the heme groups, which are resistant to dimerization. The electron transfer is visualized below, NADPH-oxidase donating an electron to be ultimately used to convert arginine and oxygen to the reaction products citrulline and nitric oxide. Tetrahydrobiopterin (BH_4) further stabilizes the dimer. Zn, zinc; Arg, arginine binding site; FMN, flavin mononucleotide; FAD, flavine adenine dinucleotide.

time, to the prosthetic heme iron of the oxygenase domain, where reduction of molecular oxygen (O_2) to O_2^- takes place. Bound L-arginine is then converted to NO and the by-product L-citrulline. This electron transfer onto heme is mediated by FAD [13]. The oxygenase is linked to the reductase domain by an inhibitory calmodulin binding domain. To be active, eNOS has to be deinhibited by the binding of two calcium-activated calmodulins, which secure the electron transfer from the reductase to the oxygenase domain (Fig. 2) [14].

Regulation of eNOS

eNOS is predominantly targeted to the sarcolemmal caveolae, which are invaginations of the plasma membrane. Here the enzyme is bound by posttranslational myristoylation and palmitoylation to caveolin-1, a resident coat protein that inhibits eNOS activity. Caveolin-1 interacts with the calmodulin (CaM) binding sites and inhibits the electron transfer from NADPH at the reductase to the heme molecule in the oxygenase domain. At a cellular level, eNOS is therefore activated by a calcium-mediated disruption of the eNOS–caveolin heteromeric complex. Caveolin-free eNOS is then translocated from the caveolae to the cytoplasm. Its enzymatic function there is greatly upregulated, including also Ca^{2+}-independent steps. As a result, the electron flow from the reductase to the oxygenase domain is initiated, and NO is produced. In addition, in the caveolae the substrate L-arginine is recycled from L-citrulline, ensuring a sufficient pool for eNOS [15–17]. In addition, eNOS activity is influenced by posttranslational modifications, such as calcium influx, and phosphorylation at various amino acid residues [18]. Calcium-activated CaM is essential for rapid enzyme activation, although in vivo eNOS may become calcium independent through a reduction in CaM dissociation from activated eNOS [19]. Phosphorylation is of key importance in regulating the overall function, activation, and, potentially, coupling of eNOS. This includes phosphorylation of calmodulin, caveolin, and amino acid residues on eNOS itself. The production of both NO and superoxide by eNOS can be modulated by protein kinase Cα (PKCα)-mediated phosphorylation of calmodulin and caveolin. Phosphorylation of eNOS at Ser1177 is pivotal in the direct regulation of superoxide versus NO generation, altering both the calcium sensitivity of the enzyme and the rate of reaction product formation [20]. In detail, direct phosphorylation of Ser1177 by adenine monophosphate kinase (AMPK) enhances eNOS activity by promoting its association with heat shock protein 90 [21]. This is also true for the uncoupled eNOS, for which Akt-mediated phosphorylation of Ser1177 markedly enhances maximal superoxide generation at low levels of calcium, making NO generation largely calcium independent [19]. However, phosphorylation at Thr495 at the CaM binding domain prevents the increase in catalytic activity of eNOS by hindering the association of CaM with eNOS [22]. In addition, phosphorylation at Ser633 in the FMN binding domain has been associated with increased enzyme activity after initial activation by Ca^{2+} influx and Ser1177 phosphorylation [18]. eNOS can also be phosphorylated at Ser114 and Ser615, but the functions of these phosphorylation sites remain controversial [23].

BH_4 is obligatory for optimal eNOS activity. First, it ensures normal function at the heme catalytic site. In the absence of BH_4, reduction of the ferric ion of the eNOS heme group translates to the formation of an Fe(II) dioxygen complex, resulting in superoxide formation. In the presence of BH_4, iron–oxy species are formed, which participate in L-arginine hydroxylation and thus in NO generation. Second, it increases eNOS affinity for L-arginine [24]. Third, it is hypothesized to play a role in the electron transfer, being converted to a BH_3 radical during the first catalytic reaction [25]. Fourth, BH_4 interacts with amino acid residues from both monomers to stabilize dimerization [26]. In addition, BH_4 can act as a modest scavenger of reactive oxygen (ROS) and nitrogen species.

BH_4 can be oxidized by ROS, leading to a reduction in vascular BH_4 and an increase in vascular BH_2 availability [27]. A lack of this crucial cofactor results in decreased eNOS function and a further increase in ROS production combined with a decrease in NO production [28,29]. In addition the oxidation product, BH_2, is a competitive BH_4 antagonist [30].

BH_4 was initially described as the oxygen acceptor and cofactor for aromatic amino acid hydroxylases, involved in neurotransmitter biosynthesis. In eNOS, however, heme is the oxygen receptor and

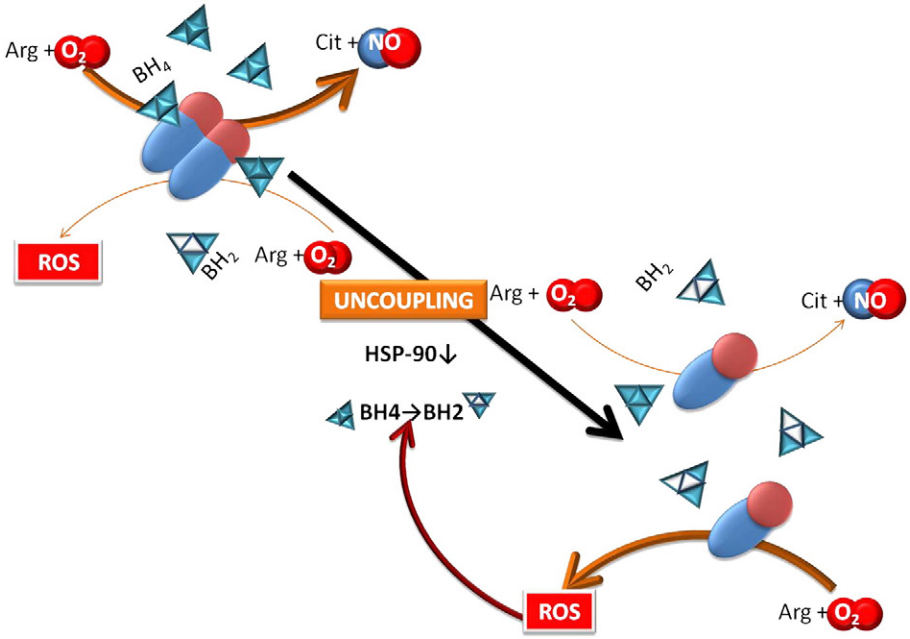

Fig. 2. Schematic overview of eNOS uncoupling. In the presence of enough BH_4, the eNOS dimer produces abundant amounts of NO (thick arrow) and a small fraction of ROS (thin arrow). BH_4 binds the oxygenase domain of eNOS and stabilizes the dimer. However, in absence of BH_4, when there is more BH_4 oxidized to BH_2, and/or when the HSP-90 concentration drops, the eNOS dimer will uncouple into two monomers, which are less efficient in the production of NO and generate large amounts of ROS (thick arrow). The reaction self-perpetuates because ROS generated by uncoupled eNOS oxidize BH_4 themselves. Arg, Arginine; Cit, citrulline; ROS, reactive oxygen species; BH_4, tetrahydrobiopterin; BH_2, dihydrobiopterin; HSP, heat shock protein.

BH_4 has different functions. BH_4 can be formed through two different pathways, i.e., a de novo and a salvage pathway (Fig. 3). The de novo synthesis of BH_4 starts with guanidine triphosphate, which is converted by guanidine triphosphate cyclohydrolase-1 (GTPCH-1) and sepiapterin reductase to BH_4. GTPCH-1 is the rate-limiting enzyme in the biosynthesis of BH_4. It is inhibited by the specific GTPCH feedback regulatory protein and is under positive feedback by phenylalanine [31]. This regulation takes place by altering the transcription levels, and thus, the production of BH_4 is largely dependent on GTPCH-1 expression. Transcription is increased by inflammatory factors such as tumor necrosis factor-α, interleukin-1, interferon-γ, and lipopolysaccharides [32]. GTPCH-1 is localized in the caveolar microdomains of the cardiomyocyte together with caveolin-1 and eNOS [33], where it provides a continual source of BH_4. GTPCH-1 expression is also determined by the GTPCH-1 haplotype [34]. Certain autosomal-dominant inherited mutations in the GTPCH-1 enzyme lead to congenital BH_4 deficiency and neurological symptoms known as dopa-responsive dystonia [35]. In mammals, the main salvage pathway progresses through dihydrofolate reductase (DHFR) and dihydropteridin reductase (DHPR). DHFR mainly activates folic acid to 5-methyltetrahydrofolate and also regenerates BH_4 from its oxidized form BH_2. DHPR uses the quinonoid form of BH_2 as substrate.

Uncoupling of eNOS

Uncoupling of eNOS can be visualized by SDS–polyacrylamide gel electrophoresis as a loss of the eNOS dimer and an increase in the monomer-to-dimer ratio [36]. The uncoupled state can thus also be interpreted as an altered quaternary structure of the enzyme.

Vasquez-Vivar et al. [30] demonstrated the effects of BH_4 on the balance between NO and superoxide formation. In absence of BH_4, eNOS becomes uncoupled and cannot reach its normal NO-generating abilities. Once eNOS is uncoupled because of decreased bioavailability of BH_4, the uncoupling propagates because uncoupled eNOS generates superoxide, which will oxidize the remaining BH_4 [27]. Superoxide may react with NO, forming peroxynitrite [37]. The increased activity of enzymes such as NADPH oxidases ignites a cascade of radical formation by producing superoxide as a kindling radical. Bursts of peroxynitrite oxidize BH_4 to BH_3, further increasing eNOS uncoupling, starting a bonfire of radical production by eNOS. Peroxynitrite can also lead to irreversible nitration of tyrosine residues on other proteins [38], causing impaired phosphorylation and enzymatic dysfunction [39].

Importantly, elevated eNOS expression, without a parallel increase in BH_4, results in eNOS uncoupling because of an imbalance between the cofactor and the enzyme [40]. Crabtree et al. [41] investigated the stoichiometry of intracellular BH_4/eNOS interactions and demonstrated a striking linear relationship between eNOS activity and cellular BH_4 levels, with eNOS uncoupling occurring when the eNOS:BH_4 molar ratio exceeded 1. Increasing intracellular BH_2 concentration in the presence of a constant eNOS:BH_4 ratio was sufficient to induce eNOS-dependent superoxide production, indicating that eNOS/BH_4 reaction stoichiometry has a tandem role with the intracellular BH_4:BH2 ratio (rather than absolute plasma concentrations of BH_4) in determining eNOS uncoupling, even when exogenous oxidative stress is absent. In another recent study, Heiss et al. [42] demonstrated that a decrease in BH_4 levels activates NF-E2-related factor, which leads to a reduction in eNOS protein levels in human endothelial cells. Thus, the stoichiometric balance between BH_4 and eNOS is maintained, eNOS is kept in a coupled state, and ROS production is reduced. Crabtree et al. [41] showed that murine endothelial cells contain large amounts of DHFR and that reduction of DHFR activity by methotrexate, or genetic knockdown of DHFR by RNA interference, resulted in oxidation of BH_4 to BH_2 with subsequent eNOS uncoupling. DHFR can regenerate BH_4 from BH_2 and preserve eNOS coupling by helping to maintain a better BH_4:BH_2 ratio, especially when both are scarce.

The essential role of L-arginine in keeping eNOS coupled is debatable. It is unlikely that plasma L-arginine levels would fall below the critical concentration for eNOS activity in vivo, as the plasma level of L-arginine is 30 times higher than the eNOS K_M

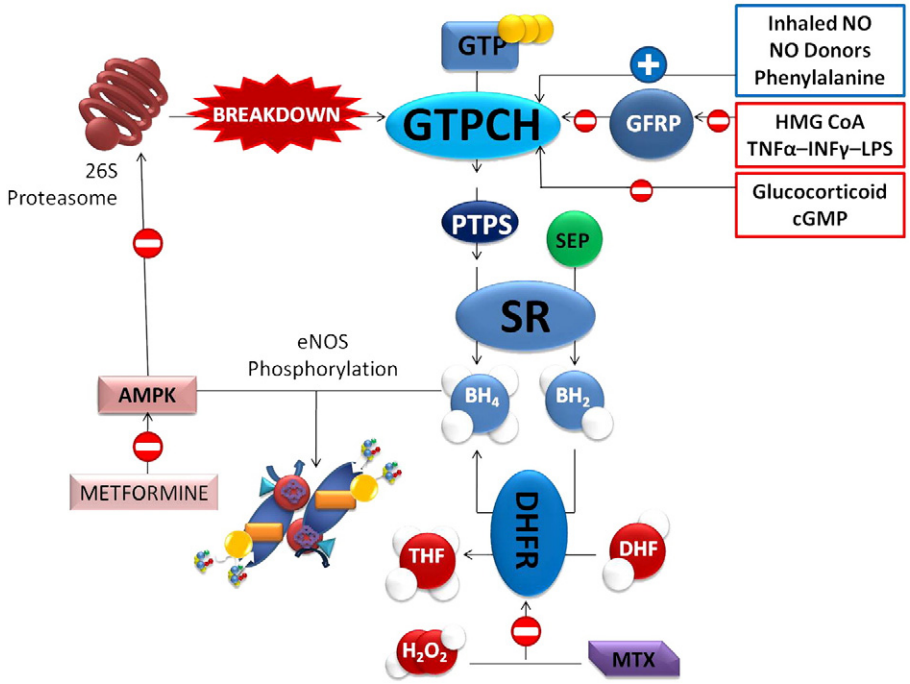

Fig. 3. Cellular pathways involving tetrahydrobiopterin (BH_4). Guanidine triphosphate cyclohydroxylase (GTPCH) is inhibited by glucocorticoids, cGMP, and GFRP (GTPCH feedback regulatory protein), which is influenced by statins and inflammatory mediators such as TNFα. Nitric oxide stimulates GTPCH, which activates sepiapterin reductase (SR) through 6-pyruvoyltetrahydropterin synthase (PTPS). Sepiapterin is converted into dihydrobiopterin (BH_2) and may be further reduced to tetrahydrofolate (BH_4) by dihydrofolate reductase (DHFR), which also reduces dihydrofolate (DHF) to tetrahydrofolate (THF). BH_4 assists in eNOS coupling, which is activated by phosphorylation by AMP-activated protein kinase (AMPK). AMPK is inhibited by the antidiabetic agent metformin, and AMPK itself inhibits the 26S proteasome, thereby inhibiting breakdown of GTPCH.

($3\ \mu mol \cdot L^{-1}$ vs $100\ \mu mol \cdot L^{-1}$) [41]. In addition, L-arginine itself is recycled by the cell [43]. An increased intracellular breakdown by arginases, however, can lead to a very local, subcellular decrease in intracellular L-arginine. As such, arginases, expressed in endothelial cells, can compete with eNOS for their common substrate [44] and can therefore also have downregulating effects on eNOS [45]. The eNOS inhibitor asymmetric dimethylarginine (ADMA) directly inhibits eNOS, and it also inhibits cellular L-arginine uptake by endothelial cells. Therefore, a decreased L-arginine:ADMA ratio can result in reduced NO formation by eNOS. Indeed, Antoniades et al. [46] demonstrated a strong inverse association between serum ADMA and levels of eNOS dimer, in both human arteries and human veins. Furthermore, increased ADMA plasma concentrations are associated with oxidative stress within the vessels and the development of endothelial dysfunction [47]. Thus rather than changes in L-arginine, changes in ADMA seem to trigger eNOS uncoupling. However, Druhan et al. [48] demonstrated that, although ADMA can uncouple eNOS and elevate ROS generation in the absence of BH_4, this is not the fact when adequate levels of L-arginine are present. Also, in this study ADMA and L-arginine elevated ROS produced by uncoupled eNOS in equal fashions. This may indicate that ADMA is not responsible for eNOS uncoupling under physiological conditions.

The ZnS_4 cluster in the eNOS oxygenase domain, formed by a zinc ion and two cysteine residues from each monomer, is positioned equidistant from each heme group and is responsible for the maintenance of the integrity of the BH_4 binding site [11]. Mutation in this cluster prevents the binding of zinc, BH_4, or L-arginine and eliminates enzyme activity [49], suggesting that stabilization of the dimer interface by the zinc thiolate center is one of the keys for catalytic activity. Exposing the isolated eNOS enzyme to peroxynitrite, a by-product of the interaction between excessive NO and $O_2^{\bullet -}$, leads to the oxidation of the zinc thiolate cluster, which uncouples eNOS. Recently, Chen et al. [50] demonstrated that the dimer stabilization induced by BH_4 does not require zinc occupancy in this thiolate cluster. Although peroxynitrite treatment induced loss of Zn binding and compromised eNOS activity, incubation with a zinc chelator did not alter eNOS activity.

Role of eNOS uncoupling in the cardiovascular system

Hypertension and heart failure

In mice, gene expression of eNOS influences vascular tone and hence is a major determinant of blood pressure regulation [51]. eNOS uncoupling in hypertension was first shown by Mollnau et al. [52] in an in vivo rat angiotensin II infusion model. Where deletion of the eNOS gene causes systemic hypertension [51], overexpression results in a reduction of blood pressure [53]. Landmesser et al. [27] demonstrated the pathogenetic role of eNOS uncoupling in DOCA salt hypertension. Later, Takimoto et al. [54] confirmed the key role of eNOS uncoupling in pressure-overload-induced ventricular remodeling using a model of transverse aortic constriction. They demonstrated that oral administration of BH_4 to wild-type mice prevented cardiac hypertrophy and dilation, improved heart function, and inhibited eNOS uncoupling with a subsequent increase in NO synthesis and a decrease in ROS generation. In addition, this group showed that pressure overload in $eNOS^{-/-}$ mice resulted in less cardiac hypertrophy, dilation, and a decrease in myocardial fibrosis because no eNOS was available to become uncoupled, and consequently, there was no detrimental NOS-dependent superoxide generation. Further, Moens et al. [55] demonstrated that oral administration of BH_4 can restore uncoupled eNOS and reverse preexisting ventricular hypertrophy, fibrosis, and myocardial dysfunction. Treatment with a general antioxidant such as Tempol had no effect on eNOS and could not produce the same positive results on preexisting ventricular remodeling as BH_4.

Diabetes mellitus

Insulin resistance induces eNOS uncoupling through increased superoxide production [56] in streptozotocin-induced diabetic LDL receptor-deficient mice. In the vessels of rats with streptozotocin-induced diabetes both upregulation and uncoupling of eNOS are observed in a PKC-dependent process [57]. This is observed despite an increase in eNOS expression, suggesting that eNOS becomes uncoupled rather than downregulated. Diabetes mellitus (DM) type 2 is associated with increased nitrotyrosine formation mediated by peroxynitrite and subsequent endothelial dysfunction [58]. In mice treated with streptozotocin, which selectively destroys the β cells of the pancreas, inducing DM, eNOS becomes uncoupled but also expressionally upregulated [59]. This results in a decrease in BH_4 levels and compensatory GTPCH-1 overexpression in the endothelium of the diabetic aorta [59,60]. However, the influence of this GTPCH-1 overexpression is limited in light of uncoupled eNOS, and a subsequent decrease in BH_4 is observed [60]. There is also evidence that GTPCH-1 is downregulated in diabetes and not compensatorily upregulated [61]. The dysfunction of endothelial progenitor cells in patients with DM 2 and the consequent lack of vascular repair have also been linked to eNOS uncoupling induced by BH_4 deficiency [62]. Recently Wang et al. [63] described that a decrease in AMPK results in abnormal activity of the 26S proteasome, leading to an accelerated destruction of GTPCH in a streptozotocin model of DM 2 in mice. In patients with DM 2, metformin, which activates AMPK, was shown to reduce mortality and improve vascular function. Inhibiting the breakdown of GTPCH-1 by the 26S proteasome via AMPK upregulation is therefore an additional pathway for maintaining normal eNOS activity.

Atherosclerosis

Oelze et al. [64] first demonstrated that eNOS uncoupling is at least partially involved in the increased ROS formation in the vessels of hyperlipidemic rabbits. Antoniades et al. [65] investigated the role of BH_4 in atherosclerosis using saphenous veins and internal mammary arteries from patients with coronary artery disease (CAD) undergoing coronary bypass surgery. They found that plasma biopterin levels are inversely correlated with vascular biopterins, which are mostly present in the endothelium. Vascular BH_4 levels were inversely correlated with vascular ROS generation and positively correlated with eNOS coupling and NO-mediated endothelial function. Plasma BH_4 levels were also positively correlated with impaired endothelial function [65] and with C-reactive protein (CRP) levels, a prototypic marker of inflammation. The direct effects of CRP on cardiovascular risk are debated and CRP may better be regarded a marker of cardiovascular disease, rather than a mediator. The pathogenetic role of eNOS uncoupling is described in Fig. 4.

Proinflammatory stimuli are sufficient to increase the amount of circulating plasma biopterins, but fail to increase biopterin levels in the endothelium itself, leading to endothelial dysfunction by eNOS uncoupling. As described above, the specific GTPCH-1 haplotype of the patient determines the levels of BH_4 in plasma and vascular endothelium [34]. This GTPCH haplotype [66] is defined by three single-nucleotide polymorphisms, rs8007267G→A in the putative promoter region, rs3783641A→T in intron 1, and rs10483639C→G in the 3′ untranslated region of the GTPCH-1 gene, and is associated with increased vascular superoxide generation by uncoupled eNOS and decreased endothelial function, independent of other risk factors for atherosclerosis. The existence of different GTPCH haplotypes and genetic differences in BH_4 availability may therefore be an explanation for the heterogeneity in patients with coronary artery disease. eNOS activity has not only been associated with risk factors for CAD, it may also be of importance in its prevention. Regular aerobic exercise reduces the risk of cardiac events, possibly through shear-stress-

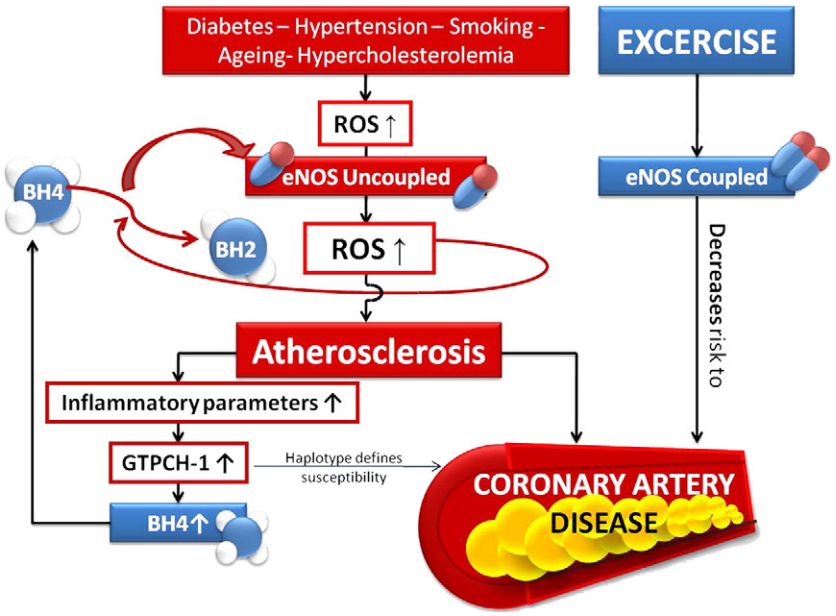

Fig. 4. Involvement of eNOS in atherogenesis. Various risk factors for atherosclerosis may lead to eNOS uncoupling, causing an increase in ROS. Oxidation of BH_4 to BH_2 is critical in this process. The enormous amounts of ROS cause endothelial damage, inflammation, and atherosclerosis. Depending on the GTPCH-1 haplotype, patients may be more susceptible to this mechanism and thus coronary artery disease. Upregulation of GTPCH-1 or external supplements of BH_4 may slow this process down or even reverse it.

induced eNOS upregulation and NO production. Regular physical exercise ameliorates peripheral NO-dependent vasodilatation in patients with chronic heart failure [67] and coronary vasomotor activity in CAD patients [68]. Shear stress activates the tyrosine kinase c-Src and initiates an intracellular cascade, which increases eNOS mRNA transcription and stability in vitro [69] through activation of a shear-stress response element on the eNOS promoter. Mice that are heterozygous for c-Src deletion no longer display a positive correlation between training and vascular eNOS expression, indicating that the c-Src pathway is pivotal in how physical training modulates eNOS function [70].

Ischemic heart disease

Oxidative stress at the cellular level plays a key role in myocardial remodeling after myocardial infarction (MI) [71,72]. Previous studies have indicated that cardiac remodeling after MI was attenuated in transgenic mice overexpressing eNOS, but deteriorated in $eNOS^{-/-}$ mice [73]. Similarly, NO can increase angiogenesis [74] and decrease cardiac fibrosis [75] after MI. The presence of eNOS attenuates left-ventricular dysfunction and remodeling in a murine model of MI by afterload-independent mechanisms, partially by decreasing myocyte hypertrophy in the myocardium [76]. In addition, Masano et al. [77] demonstrated in a rat model of MI that the ventricular remodeling process after MI was associated with increased superoxide production from the noninfarcted myocardium. Yaoita et al. [78] reported, using a rat model, that an increase in eNOS activity by supplementation of BH_4 lowers the myocardial activation of neutrophils after surgically created coronary ligation and subsequent ischemia, protecting both endothelial cells and cardiomyocytes from myocardial inflammation. This was attributed to an eNOS-dependent improvement in coronary arteriole endothelial function and therefore in myocardial perfusion. Consequently, modulating eNOS by administration of BH_4 can be considered a potential therapeutic agent to counteract the damaging effect of ischemia through improved coronary perfusion and to attenuate ventricular remodeling and therefore preserve cardiac function. Furthermore, the occurrence and subsequent alleviation of eNOS uncoupling after MI were indicated by a reduction in the eNOS dimer/monomer ratio and its restoration by exogenous BH_4 [55]. Supplementation with the BH_4 precursor sepiapterin or the synthetic BH_4 analogue 6-methyltetrahydropterin both improves response to endothelium-dependent vasodilators in coronary arteries as was demonstrated in vivo in a pig model of coronary occlusion and subsequent reperfusion [79]. However, in another paper by Vasquez-Vivar et al. [30], it was demonstrated that sepiapterin may also uncouple eNOS by antagonizing BH_4. Therefore, it may not always work.

Smoking

Cigarette smoke contains high amounts of radicals such as NO, O_2^-, and $ONOO^-$. Reduced availability of BH_4 because of oxidation by these radicals may cause eNOS uncoupling and this process, at least in part, contributes to the endothelial damage and dysfunction in chronic smokers. Supplementation of BH_4 can improve vasodilatator response in the brachial artery of chronic smokers. However, the mere antioxidant properties of BH_4 may also be responsible for this effect. Heitzer et al. [80] found that BH_4, but not tetrahydroneopterin (also a reduced pteridine), could modify Acetylcholine-induced changes in forearm blood flow of chronic smokers, which points to a specific effect of BH_4 on eNOS rather than its action as a nonspecific antioxidant.

Nitrate tolerance

Nitrate tolerance is defined as a decline in the vasodilatory effects of nitroglycerin (NTG) after a period of its continuous administration. Oxidative stress in NTG-exposed blood vessels reduces NO bioavailability, resulting not only in compromised relaxation, but also in reduced sensitivity of the vessels to endothelium-dependent vasodilatators. The source of NTG-induced superoxide is still unknown, but uncoupled eNOS is considered to be a potential candidate in addition to components of the mitochondrial respiratory chain and NADPH oxidases. Münzel et al. [81] demonstrated in a model of NTG-treated rats that increased endothelial O_2^- production is responsible for nitrate tolerance after long-term nitrate treatment. Following these observations they showed that eNOS protein and RNA expression is significantly increased after NTG treatment. In nitrate-tolerant vessels the increased production of ROS was caused by uncoupling of higher

expressed eNOS and this process depended on phosphorylation by PKC. Ikejima et al. [82] demonstrated significantly reduced BH_4 levels possibly leading to eNOS uncoupling in aortic segments obtained from rabbits that had been continuously treated for 7 days with transdermal NTG patches. However, this could not be demonstrated by Schmidt et al. [83] using an established guinea pig model of nitrate tolerance. Furthermore, Gori et al. [84] demonstrated that administration of folic acid can prevent NTG-induced endothelial nitric oxide dysfunction and nitrate tolerance in healthy male volunteers.

Cardiovascular aging

It is well documented that skeletal muscle vascular conductance and endothelium-dependent vasodilatation are reduced with aging. Aging in rats causes a decline in eNOS activity, which may contribute to the development of hypertension [85]. eNOS uncoupling and a decrease in NO bioavailability may contribute to this aging-related decline in flow-induced vasodilatation and subsequent increase in blood pressure. In humans, a single dose of oral BH_4 could restore endothelium-dependent dilatation in the conduit brachial artery of sedentary older men [86]. In addition, Sindler et al. [87] found that age-related reduction in BH_4 occurred in conjunction with a decline in flow-induced NO signaling and an increase in superoxide production by eNOS uncoupling. Exercise training prevented this age-related loss of BH_4 and improved NO bioavailability by balancing accelerated NO and ROS production.

eNOS uncoupling in pulmonary diseases

Pulmonary artery hypertension

Chronic hypoxia is one of the main causes of sustained pulmonary artery hypertension (PAH) in patients with chronic obstructive lung disease or congestive heart failure or during cardiopulmonary bypass surgery [5,88]. Impairment of NO production is regarded as a possible mechanism in the pathogenesis of PAH [89], as $eNOS^{-/-}$ mice are more susceptible to hypoxia than wild-type controls [90]. In rats with hypoxia-induced PAH, eNOS activity, but not eNOS expression, was lowered, indicating that eNOS is inactivated at a posttranslational level. Murata et al. speculated that eNOS inactivation may be caused by a lack of dissociation between eNOS and caveolin-1, inhibiting the translocation of eNOS to the cytosol and impairing Akt-mediated eNOS phosphorylation at Ser1177 [91]. This is in contrast to systemic hypertension, which showed eNOS uncoupling as the critical cause of inactivation of the decrease in the amount of NO produced [27]. In addition, Xu et al. [45] described a decrease in the eNOS substrate L-arginine [45] from increased arginase II activity during PAH. Furthermore, an increase in the endogenous eNOS inhibitor ADMA during PAH has been described [92]. Elevation of ADMA is indeed a potential pathogenetic mechanism for pulmonary hypertension in patients with congenital heart disease [93]. ADMA is mostly elevated in the pulmonary vascular endothelium of PAH patients in areas of intimal fibrotic and plexiform lesions, compared to healthy patients.

Acute lung injury and acute respiratory distress syndrome

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are acute inflammatory states, which are characterized by a sudden onset of dyspnea, severe hypoxemia, neutrophil pulmonary sequestration, and pulmonary edema secondary to disruption of pulmonary capillary integrity. They remain significant causes of morbidity and mortality in hospitalized patients [94]. Sharma et al. [95] used a lipopolysaccharide (LPS)-induced mouse model of ALI to demonstrate that LPS increases the level of ADMA in endothelial cells. ADMA may be responsible for NOS uncoupling transiently at early stages of ALI. Peak superoxide production occurred 2 h after exposure to LPS. It was not possible to determine which NOS isoform was predominately responsible for this increase in NOS-derived superoxide. Previously, LPS treatment had been shown to reduce mRNA expression and decrease eNOS protein expression in bovine endothelial cells [96] and 12 h post-LPS treatment in the mouse lung [97]. As ADMA is associated with eNOS uncoupling [46], it may be possible that eNOS is involved in superoxide and peroxynitrite generation in ALI, leading to protein nitration associated with lung leak in ALI. Peroxynitrite scavengers reduced the nitrated protein levels and decreased capillary leakage. However, further investigation into the individual role of eNOS in ALI is warranted.

Hypoxia associated with proinflammatory mediators in ARDS augments the formation of ROS by increased NADPH oxidase expression. In ARDS an increase in eNOS expression is observed, which may be caused by mechanical ventilation leading to Akt-mediated phosphorylation [98]. The concomitant upregulation of eNOS and NADPH oxidase results in increased $ONOO^-$ formation and may advance ARDS by promoting tissue damage and inflammation [99]. Therefore, specific antioxidants targeting eNOS uncoupling and/or its triggers could be an interesting therapeutic approach to ARDS.

eNOS uncoupling during thoracic surgery

As described above, NO is not only a vasodilator, it also inhibits platelet aggregation, thrombus formation, and smooth muscle cell proliferation and therefore may interfere with cardiovascular outcome. A lack of NO due to eNOS uncoupling may negatively affect postoperative patient outcome. The effects of NO metabolism and eNOS uncoupling have been thoroughly investigated in coronary artery bypass graft surgery (CABG) and lung transplantation.

CABG surgery

Conventional stripping of the saphenous vein is associated with more damage to the endothelium and the tunica adventitia and lower eNOS levels in venous endothelium than atraumatic harvesting, by which the surrounding tissue is spared [100]. Impaired NO synthesis due to endothelial damage during graft harvesting may cause early graft failure [101]. Together with an increase in growth factors after ischemia and reperfusion (IR), this diminished NO effect results in neointimal hyperplasia, reduced lumen diameter, and decreased coronary blood flow, which are associated with later stages of graft failure. This may influence the long-term outcome of CABG procedures. Modulating eNOS uncoupling in the cardioplegia solution may also be an adequate approach to preventing CABG failure by endothelial dysfunction. Maintenance of normal NO homeostasis is an important factor in protecting the graft and the heart from IR injury during cardioplegia [102]. Interindividual differences in recuperation from CABG procedure are not dependent on polymorphisms of the eNOS gene, as these do not have any influence on early postoperative hemodynamics after cardiac surgery [103]. Therefore, addition of BH_4 and L-arginine may prevent eNOS uncoupling, decrease inflammation, and prevent graft failure [102].

Lung transplantation

Lung preservation, transplantation, and reperfusion lead to reduced endogenous NO production in lung tissue, indicated by a decreased amount of exhaled NO [104]. Liu et al. [105] investigated the effects of eNOS on lung transplantation surgery in the rat. They demonstrated a reduction in eNOS mRNA during lung transplantation, which was possibly caused by a lower ability of the endothelial and lung epithelial cell to respond appropriately to ischemia and reperfusion. Transplanted lungs had decreased bioavailability of NO. IR may have a direct effect on eNOS synthesis and degradation, contributing to injury in lung transplants. Administration of the NO

donor NTG prevented reperfusion-induced lung injury such as endothelial injury in alveolar and bronchial epithelial cells in an ex vivo rat lung perfusion model [106]. Administration of the eNOS cofactor BH_4 during lung allograft reperfusion in a pig model of lung transplantation reduced posttransplantation lung edema and oxygen-derived free radical injury in the graft [107]. Exogenous administration of NO by inhalation from cylinders containing nitrogen for 84 h on average (15–217 h) after bilateral lung transplantation reduced early severe graft dysfunction, as indicated by a decrease in hypoxia and PAH. Also, these patients needed shorter postoperative mechanical ventilation, had fewer airway complications, and had a decrease in general mortality [108].

Wound healing

Vasodilation and hyperemia of existing vessels, both NO-mediated processes, are coincident with angiogenesis [109]. Newly formed vessels deliver the necessary metabolic substances to the regenerating tissue to assist wound repair. Closure of the wound is mediated by both wound contraction and wound reepithelialization. The effects of vascular endothelial growth factor (VEGF), one of the key mediators of angiogenesis, are mediated by NO [110]. VEGF upregulates eNOS protein levels and NO production [111], and activation of VEGFR-2 results in upregulation and phosphorylation of eNOS and increased NO production by inducing a peak in intracellular Ca^{2+} levels [112]. In $eNOS^{-/-}$ mice, closure of excisional wounds where a square piece of skin was removed was significantly impaired, and healed cephalad-to-caudal incisional wounds developed less tensile strength [113]. This may be of therapeutic importance in such diseases as diabetes mellitus in which a deficit in VEGF expression is part of the associated defect in wound healing [114].

Modulating the eNOS pathway

L-Arginine

L-Arginine is the primary substrate for endothelial NO production by eNOS and supplementation can improve endothelial-derived dilatation, decrease adhesion of platelets and monocytes, and reduce systemic and pulmonary artery pressure [115]. A lack of L-arginine may lead to eNOS uncoupling. L-Arginine is broken down by arginases that compete with eNOS for their common substrate [44]. In pulmonary endothelium of patients with pulmonary artery hypertension [45] and in endothelial cells of the corpus cavernosum of diabetes patients [49], arginase expression indeed seems to be upregulated. Therefore, the L-arginine deficiency in endothelial dysfunction can be explained as a very local and relative lack of this amino acid, situated in the direct vicinity of eNOS by an upregulation of L-arginase [49]. This could be a partial explanation of the beneficial effect of L-arginine supplementation observed in some, but not all, clinical studies [116]. In addition, L-arginine can also serve as a direct radical scavenger [117] and compete with the endogenous eNOS inhibitor ADMA [46].

Three days of oral L-arginine administration to young men with CAD showed improvement in endothelium-dependent vasodilatation and reduced monocyte adhesion [118]. In patients with heart failure, oral administration of L-arginine was shown to improve renal function [119], vascular function, and cardiac output [120]. In hypercholesterolemic patients administration of L-arginine improved endothelium-dependent forearm dilatation [121], just as effectively as statin therapy [122]. In addition, some patients with PAH benefit from intravenous infusion of L-arginine, as it increases L-arginine and L-citrulline concentrations and has a potent short-term vasodilating effect, equal to prostacyclin but better tolerated, reducing pulmonary artery pressure [123]. However, most of the positive studies investigating L-arginine supplementation were rather short. In contrast, L-arginine may not be beneficial if given chronically, as shown in the VINTAGE MI study [124]. Nevertheless, this study has been criticized for serious flaws [125]. A meta-analysis concluded that short-term L-arginine treatment improved endothelial function in only those individuals with endothelial dysfunction, as determined with flow-mediated vasodilation [116].

BH_4

BH_4 is an FDA-approved therapy for some forms of phenylketonuria, in which there is a deficiency in the hepatic enzyme phenylalanine hydroxylase. Furthermore, BH_4 is of critical importance in stabilizing the eNOS dimer, keeping it in coupled form, allowing eNOS to function appropriately. In addition, oral administration of BH_4 after acute myocardial infarction induction in rat heart attenuates the remodeling and preserves cardiac function by decreasing superoxide generation [77]. Reduction of the eNOS dimer/monomer ratio after MI in these rats and its restoration by exogenous BH_4 indicated the occurrence and subsequent alleviation of eNOS uncoupling. BH_4 has been shown to improve endothelial dysfunction in patients with DM 2 [126], heart transplant patients treated with cyclosporine A [127], and patients with inflammatory disorders such as rheumatoid arthritis [128]. BH_4 was also found to lower blood pressure [129] but longer term studies have failed to show any significant benefit [130]. BH_4 is temperature and light unstable, very hygroscopic, and quickly oxidized, as it is an antioxidant, and therefore difficult in practical use as a chronic drug treatment. The BH_4 analogue sapropterin hydrochloride (BioMarin Pharmaceuticals, CA, USA) does not have these stability problems.

Folic acid

Folic acid has direct superoxide scavenging effects [131] and increases the bioavailability of BH_4 in the vasculature by preventing its oxidation [132] and ensuring eNOS coupling. Chronic administration of 5-MTHF, the active form of folic acid, improves endothelial function in patients with systolic heart failure [133], reverses eNOS dysfunction and uncoupling in NTG-treated patients [84], and can restore endothelial function in hypercholesterolemic patients [134]. High-dose folic acid administration to patients with CAD showed no more improved vascular function compared to a low-dose treatment of folic acid [135]. No human data yet are available about the effects of folic acid on myocardial dysfunction.

Other eNOS-modulating agents

Statins have been reported to increase eNOS stability and its expression on the cell membrane in addition to their well-known LDL-lowering effect [136]. Statins activate adenosine receptors A_1, A_{2A}, and A_{2B}, which leads to Akt-mediated eNOS phosphorylation [137]. The cardioprotective effects of angiotensin converting enzym inhibitors and angiotensin receptor blockers (ARBs) are also mediated by eNOS-generated NO. ACE inhibitors and ARBs activate eNOS by stimulation of a PI3-kinase/Akt pathway [138]. Both statins and modulators of the rennin–angiotensins system also inhibit NADPH oxidases, potentially resulting in reduced eNOS uncoupling. Estrogens increase eNOS phosphorylation and therefore its activity. This may be an explanation for the lower incidence of atherosclerosis in premenopausal women. Estradiol directly activates eNOS by binding on the estrogen receptor and subsequently activates the cGMP-dependent protein kinase G pathway, which phosphorylates the eNOS enzyme [139]. This way, estradiol is a protective agent in IR injury, as was demonstrated in lung tissue after trauma–hemorrhage [140]. A therapeutic possibility for treatment of PAH is sildenafil, which increases pulmonary vasodilatation [141]. Chronic use reduces pulmonary arterial pressure in primary pulmonary hypertension, inhibits

remodeling of the vessels after hypoxia [142], and also reduces pulmonary hypoxic vasoconstriction in healthy volunteers by raising cGMP [143], as reflected in higher plasma concentrations. Sildenafil synergistically enhances the effectiveness of NO inhalation because they both raise cGMP levels, as shown by a longer lasting and quantitatively higher pulmonary vasodilatation, in both ventricular insufficiency and pulmonary arterial hypertension. High doses of vitamin C can acutely improve endothelial dysfunction. Its antioxidant effects stabilize BH_4, reducing the formation of its oxidation product BH_2 [144], and facilitate the recycling of BH_4, independent of superoxide scavenging [39]. Another eNOS modulator is AVE9488, a so-called eNOS enhancer, which may serve as a new cardioprotectant, as it improves left-ventricular remodeling and function and diminishes endothelial dysfunction after experimental MI in rodents [145,146]. No clinical data from AVE9488 are available yet.

Conclusion

During the past few years, eNOS uncoupling, and especially eNOS-dependent superoxide generation, has been suggested as having a major role in the pathogenesis of many cardiovascular and pulmonary diseases, such as systemic and pulmonary artery hypertension, heart failure, and ischemia/reperfusion injury. More research is needed to further explore the triggers (i.e., sources of superoxide) that initiate eNOS uncoupling. The possibility of targeting specific sources of ROS has paved the way for various new therapeutic approaches. Recent data have shown that by specifically targeting eNOS-dependent ROS generation, eNOS uncoupling can not only be prevented but also be reversed. Modulating eNOS uncoupling by eNOS modulators such as BH_4 and folic acid has been proven to be effective in the human vasculature. More focused research is needed to extrapolate the interesting preclinical data on these eNOS modulators to the human myocardium and lung.

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